

**Article principal
recouvrant en partie**

la THÈSE présentée

pour l'obtention

du grade de DOCTEUR ES-SCIENCES

A L'UNIVERSITÉ DE PARIS VI

par

Jacques LAVAL

Sujet de la thèse : Etude de la désoxyribonucléase acide de l'hépatopancréas *d'Helix*

soutenue le 30 Avril 1970 devant la commission d'examen.

MM. F. GROS	Président
F. CHAPEVILLE	Examineur
G. BERNARDI	Examineur
C. PAOLETTI	Membre invité

N° d'enregistrement
au C.N.R.S. A.O. 4237

Article principal
recouvrant en partie

la THÈSE présentée

pour l'obtention

du grade de DOCTEUR ES-SCIENCES

A L'UNIVERSITÉ DE PARIS VI



par

Jacques LAVAL

Sujet de la thèse : Etude de la désoxyribonucléase acide de l'hépatopancréas d'*Helix*

soutenue le 30 Avril 1970 devant la commission d'examen.

MM. F. GROS	Président
F. CHAPEVILLE . . .	Examineur
G. BERNARDI	Examineur
C. PAOLETTI	Membre invité

Purification and Properties of an Acid Deoxyribonuclease from the Snail *Helix aspersa* Müll.[†]

Jacques Laval* and Claude Paoletti

ABSTRACT: An acid deoxyribonuclease is isolated from the hepatopancreas of common snail *Helix aspersa* Müll. It is specific for DNA. The activity of the enzyme was purified 1100-fold by an eight-step procedure including dichloromethane treatment, streptomycin and ammonium sulfate precipitation, chromatography on DEAE-cellulose, Sephadex G-100, DEAE-Sephadex, and P-cellulose. The preparation is homogeneous as judged by electrophoresis on polyacrylamide gel. The enzyme activity is obtained free of contaminating acid or alkaline phosphomonoesterases, exonuclease, and unspecific phosphodiesterase. The enzyme does not require divalent cation for activity and has a pH optimum of 5.6. Ionic

Sequencing of DNA which has not yet been achieved requires endodeoxyribonucleases specific for particular sequences of DNA. Several restriction enzymes could now be used for this purpose; but, being very specific (for example, 6 base pairs recognized by the *Hemophilus influenzae* enzyme) (Kelly and Smith, 1970) they yield very long pieces of DNA, still too long for sequence analysis.

Consequently, there is an urgent need for endonucleases which could discriminate among base sequences so as to degrade DNA in small oligonucleotides enriched in nonhydrolyzable sequences. The present and accompanying papers describe an endonuclease extracted from the hepatopancreas of the common snail (*Helix aspersa* Müll.) which partially fulfills these conditions. Moreover, this enzyme displays a pH optimum which warranted a comparison of its main properties with those of the acid DNases studied by Cordonnier and Bernardi (1968), among which several could be of great significance: they are found in all higher organisms thus far investigated (Cordonnier and Bernardi, 1968), suggesting an important function, still unknown, common to all eukaryotic cells; moreover, they obey an allosteric mechanism of control of their activity (Bernardi, 1968; Lesca and Paoletti, 1969). They differ from all the other known nucleases found in bacteria and virus since they are active in an acid pH range and do not require divalent cations.

A careful investigation of the properties of the snail enzyme, which has been purified to homogeneity and is devoid of any nucleolytic enzyme contamination, reveals that besides sharing common properties with other acid DNases, it differs from them in several ways.

Furthermore the physical and chemical properties have

strength is critical for maximum activity. The isoelectric point of this protein is 5.9; $s_{20,w}$ is 3 S, its molecular weight is 30,000 as determined by gel filtration, $K_m = 1.3 \times 10^{-5}$ M, and the apparent energy of activation is 11,300 cal/mole. Fifty per cent of the enzymatic activity is lost in 10 M urea after 3 min. Heating the enzyme at 60° for 9 min reduces enzymatic activity to half. The enzyme is not inhibited by polyribonucleotides at its optimum pH but they are inhibitors at lower pH's. This acid *Helix* endodeoxyribonuclease is different by all criteria tested from splenic acid endonuclease of beef.

been investigated in details in order to correlate this DNase with the others already described.

Materials and Methods

Nucleic Acids. [methyl-³H]DNA is extracted according to Laval *et al.* (1970) from *Escherichia coli* Hfr Uvil 1858 grown on M 63 medium, containing 5 mCi per liter of [methyl-³H]-thymine. Its specific activity is 10⁷ cpm/μmole of DNA (all concentrations are expressed in nucleotides of DNA). RNA content is less than 3% as estimated with the orcinol method (Défrance and De Lesdain, 1962); protein as determined by the Lowry *et al.* (1951) procedure represents less than 1% of the DNA. Upon centrifugation on sucrose gradient, it sediments as a sharp single peak at 20 S.

Total rat liver RNA is prepared according to Hiatt (1962) and contains the three well-defined fractions (28, 17, and 4 S) found after centrifugation in the analytical Spinco Model E. RNA concentrations are determined by spectrophotometry at 260 nm, assuming that a 1-μg/ml solution has an absorbance of 0.030 (Hiatt, 1962). Yeast RNA is a gift from the Choay Laboratories, Paris. Poly(A), poly(U), poly(C), and poly(I) are purchased from Miles Research laboratories.

Enzymes and Chemicals. Splenic endonuclease of beef is prepared according to the procedure described by Bernardi and Griffe (1964) for hog spleen endonuclease. Electrophoretically pure chymotrypsin and pancreatic RNase (fraction D of Taborsky, 1959) are a generous gift from Dr. E. Sach. Crystalline horse heart cytochrome *c* (type V) and soya bean trypsin inhibitor are obtained from the Sigma Chemical Co. Bovine serum albumin fraction V is an Armour product.

DEAE-cellulose (Cellex D 0.86 mequiv/g) is purchased from Bio-Rad Laboratories, Richmond, Calif.

DEAE-Sephadex A-50 (3.5 ± 0.5 mequiv/g) and Sephadex G-100 (Pharmacia, Uppsala, Sweden) are treated before use according to the manufacturer's instructions. Cellulose phosphate, Whatmann Chromedia PI (7.4 mequiv/g), is obtained from W. and R. Balston Ltd. and treated according to Paul and Lehman (1966) before use. Bis(*p*-nitrophenyl)phosphoric

[†] From the Unité de Biochimie et Enzymologie, Institut Gustave-Roussy, 94-Villejuif, France. Received May 15, 1972. This work is a contribution for the Laboratoire n° 147 associé au C.N.R.S. This work has been aided by the Institut National de la Santé et de la Recherche Médicale, Paris, Ligue Nationale contre le Cancer, Paris, and Commissariat à l'Energie Atomique, 91-Gif-sur-Yvette, France. A preliminary account of this work was presented at the Sixth Meeting of the Federation of European Biochemical Societies, Madrid, April 1969.

acid (sodium salt) is purchased from the British Drug House, England, and sodium *p*-nitrophenylphosphate from Merck-Darmstadt, Germany. Streptomycin sulfate is a gift from Dr. Dupuis, Rhône-Poulenc, France. Ammonium sulfate (enzyme grade) and sucrose are purchased from Mann Research Laboratories. All the reagents used are analytical grade, purchased either from Prolabo, Paris, France, or from Merck, Darmstadt, Germany.

Assay Methods. *Helix* DNase ACTIVITY. General Method. This assay measures the conversion of native DNA labeled [*methyl*-³H]thymine to acid-soluble oligonucleotides. The incubation mixture (0.5 ml) contains 50 μ moles of Na acetate buffer (pH 5.6)–15.8 nmoles of DNA–enzyme. All the determinations are performed at 37° unless otherwise stated. Dilutions of enzyme are made in 0.05 M acetate buffer (pH 5.6) containing 0.05% bovine serum albumin. After 10 min at 37°, 0.5 ml of cold 0.5 N perchloric acid is added, then, 2 min later, 0.2 ml of solution of serum albumin (1 mg/ml) is added. After 5 min at 0°, the precipitate is removed by centrifugation for 10 min at 12,000g, and 0.1 ml of supernatant is poured into a counting vial. Finally, 3 ml of ethanol and 10 ml of scintillator solution (4 g of 2,5-diphenyloxazole–100 mg of *p*-bis[2-(5-phenyloxazolyl)]benzene in 1 l. of toluene) are added. Radioactivity is determined in a Packard Tri-Carb liquid scintillator spectrometer. The blank reaction in which the solution for dilution of the enzyme is substituted to the enzyme always yields less than 0.3% of the acid-precipitable material as acid-soluble counts. The amount of acid-soluble oligonucleotides released is proportional to the amount of enzyme added between 5 and 75% of substrate hydrolysis at 37°.

A unit of enzyme activity is defined as the amount of protein liberating 1 nmole of acid-soluble oligonucleotide in 1 min at 37° in the conditions described above. One unit introduces 69 endonucleolytic breaks per minute per 1×10^6 of DNA molecular weight as described in the following paper (Laval and Paoletti, 1972).

An alternative assay used to determine the ionic requirements of this enzyme measures the collapsing of the secondary structure of the DNA through the fluorescence of the complex DNA–ethidium bromide (Le Pecq and Paoletti, 1966). The incubation mixture (15 ml) contains 1.2 mmoles of thymus DNA–1.5 mmoles of acetate buffer (pH 5.6); after preincubation for 5 min at 37° with occasional stirring to allow equilibration of temperature, 1 ml of enzyme dilution is added. Every 5 min, 3-ml aliquots are transferred into 1 ml of 0.0375 M Tris solution containing 0.0825 M sodium citrate and 50 μ g/ml of ethidium bromide. Fluorescence intensity is determined at room temperature with a spectrophotometer Zeiss PM QII equipped with the fluorescence attachment ZFM4 and activity calculated from the slope of the curve IF¹ vs. time (see Table I).

PHOSPHOMONOESTERASE (PMase) ACTIVITIES. Phosphomonoesterase activities are assayed by measuring the liberation of *p*-nitrophenol from *p*-nitrophenyl phosphate according to Chersi *et al.* (1966) either in 0.1 M acetate buffer (pH 4.3) or in 0.1 M glycine buffer (pH 9.1)–0.01 M MgCl₂.

PHOSPHODIESTERASE ACTIVITY. Phosphodiesterase activity is assayed on bis(*p*-nitrophenyl) phosphate in 0.1 M acetate buffer (pH 5.6) according to Privat de Garilhe and Laskowsky (1955).

RIBONUCLEASE (RNase) ACTIVITY. RNase activity is assayed

TABLE I: Effect of Organic and Inorganic Salts on Endonuclease Activity.^a

Tested Compound	Concentration Giving 50% Inhibition (μ mole/ml)
CaCl ₂	16
MnCl ₂	7.5
Mg(CH ₃ O) ₂	6
CoCl ₂	3.75
NaF	2.6
LiCl	80
KCl	86
NaCl	80
C ₆ Cl	70
EDTA	55
NH ₄ Cl	80
(NH ₄) ₂ SO ₄	30
KH ₂ PO ₄ –K ₂ HPO ₄	28

^a Activities have been determined in 0.1 M acetate buffer (pH 5.6) by the ethidium bromide test described in Methods. The table lists the concentration of the salt needed to give 50% inhibition of endonuclease activity.

in 0.1 M acetate buffer (pH 5.0) according to Schneider and Hogeboom (1952).

In order to detect trace contamination of DNase by RNase S values of the 3 fractions of rat liver RNA are measured in the analytical ultracentrifuge after having been incubated with the purified enzyme at a concentration twice that used for the inhibition experiments by polyribonucleotides.

Sedimentation Analysis of Proteins. Sucrose gradient centrifugation is carried out according to Martin and Ames (1961) in a Spinco LHT preparative ultracentrifuge at 4°. A linear molarity gradient (4.3 ml) is obtained from 5 and 20% sucrose solutions in 0.05 M acetate buffer (pH 5.6) with a gradient maker device and a polystaltic pump (Buchler, Fort Lee, N. Y.). Beef heart cytochrome *c*, which absorbs selectively at 415 nm, is added as reference protein to the enzyme solution to be layered on the top of the sucrose gradient. After centrifugation, the bottom of the tube is punctured, and 32 ± 1 fractions are then analyzed for endonuclease activity and cytochrome *c* content. Linearity of the sucrose gradient is checked with a Zeiss refractometer.

Isoelectric Point Determination. The pH gradients are generated with carrier ampholytes (Ampholine) in the LKB 8100 electrofocusing column of 110 ml, purchased from LKB Produkter AB, Stockholm, Sweden, and used as described by the manufacturer. The voltage depends on the pH range; values will be given for each experiment. Water at 4° is circulated through the mantle during the experiment. Fractions of 1.6 ml are collected at flow rate of 1.6 ml/min. pH measurements are performed with a Tacusel pH meter at 0°.

Acrylamide Gel Electrophoresis. Preparations of *Helix* endonuclease of different specific activities are examined by acrylamide gel electrophoresis at 4°, as described by Ornstein (1964) and Davis (1964); 7.5% pore gels are used in Tris-glycine (pH 8.3). Voltage is 160 V and current 3 mA/tube for about 120 min at 4°. Bromophenol marked the advancing front which is always ahead of the protein bands. Gels are

¹ Abbreviation used is: IF, fluorescence intensity.

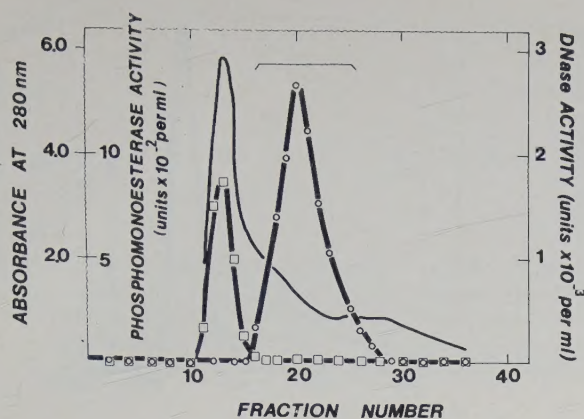


FIGURE 1: Chromatography of fraction V on Sephadex G-100: (—) absorbance at 280 nm (left scale); (○—○) endonuclease activity (right outer scale); (□—□) alkaline phosphomonoesterase activity (left inner scale). Fractions between the brackets are processed further. (For details, see text.)

stained with Coomassie Blue and destained electrophoretically. They are photographed, and then the film is scanned with the Joyce Loebel microdensitometer.

Protein Determination. Protein concentration is determined by the method of Lowry *et al.* (1951) and in effluent solutions from chromatography columns, by the absorbance at 280 nm.

Miscellaneous. Dialysis tubing is boiled in 10% sodium carbonate containing 0.001 M EDTA for 10 min and washed with cold, distilled water; this cycle is repeated; the bags are kept at 4° in a 50% solution of ethanol. Molarity gradients are checked with a Zeiss refractometer.

Results

Purification and Criteria of Purity of *Helix* Endonuclease.

PREPARATION OF EXTRACTS. The snails, *Helix aspersa* Müll., are obtained from L. Payan 84-Mazan, France; it is advisable to prepare the enzyme from animals just collected at the end of the summer in order to obtain extract of a maximum specific activity.

Snails (4 kg) are washed four times in 10 l. of 0.15 M NaCl in order to remove a heavy viscous slime and five times in 10 l. of distilled water.

The shells of the snails are broken and the hepatopancreas excised; these organs are collected in a cooled receptacle. All the subsequent operations are performed at 4°. The hepatopancreas juice is discarded from the gland. Hepatopancreas (378 g) are collected, mixed with 378 ml of chilled 0.01 M acetate buffer (pH 5.6), and homogenized for 4 min with the Ultra-Turrax at full speed. The temperature of the mixture is not allowed to rise above 10°. The resulting homogenate is centrifuged at 17,000g for 15 min in a Servall RC2 centrifuge. The supernatant (615 ml) is collected. The extraction procedure is repeated twice on the pellets, the first time with 260 ml of buffer and the second time with 176 ml. The supernatants are pooled (fraction I: 1045 ml, 534 units/ml, 30.4 mg of protein per ml) (see Table II).

DICHLOROMETHANE TREATMENT. This step is included in the purification procedure in order to remove lipid contaminants (Sih *et al.*, 1963) which otherwise would interfere with chromatographic purification.

Fraction I is divided into 300-ml portions to which are added 60 ml of precooled CH_2Cl_2 at -20°; the two layers

TABLE II: Purification Procedure.

Step		Volume (ml)	Total Protein (mg)	Total Units ($\times 10^{-4}$)	Specific Activity
I	Extract	1045	31,780	55.8	17.7
II	Dichloromethane treatment	970	20,560	40	21.7
III	Streptomycin supernatant	1090	12,420	29.6	24.3
IV	Ammonium sulfate dialysate	133.5	4,270	23.6	55
V	DEAE-cellulose and ammonium sulfate precipitation	9.5	528	16	310
VI	Sephadex G-100 chromatography	116	92.8	12.4	1,400
VII	DEAE-Sephadex chromatography	61.5	32.8	9.4	2,880
VIII	Phosphocellulose chromatography	29	0.84	1.6	19,200

are vigorously shaken for 30 sec. The resulting emulsion is immediately centrifuged for 15 min at 17,000g, and the aqueous layer is recovered. Each portion of fraction I is treated in the same way. The resulting solution is fraction II (970 ml, 413 units/ml, 21.2 mg of protein per ml); it is brown and limpid.

STREPTOMYCIN PRECIPITATION. To fraction II is added 175 ml of a chilled, freshly prepared 5% (w/v) solution of streptomycin sulfate over a period of 125 min with continuous stirring. The resulting suspension is stirred for an additional 10 min, then centrifuged for 10 min at 17,000g. The supernatant fluid (fraction III: 1090 ml, 272 units/ml, 11.4 mg of protein per ml) is immediately subjected to ammonium sulfate fractionation.

AMMONIUM SULFATE FRACTIONATION. Ammonium sulfate (308 g) is added with continuous stirring to fraction III over a 1-hr period. The suspension is allowed to stand for 15 min and is centrifuged. To the supernatant is added, under continuous stirring, 319 g of ammonium sulfate over a 90-min period. The precipitate is allowed to form for 1 hr, then centrifuged. The resulting precipitate is dissolved in 50 ml of water and titrated to pH 7.75 (at 4°) by addition of 0.5 M Tris and dialyzed for 14 hr against 4 changes of 2 l. of 0.01 M Tris buffer (pH 7.75) (fraction IV: 133.5 ml, 1770 units/ml, and 32 mg of protein per ml; pH 7.5 at 25°).

In fraction IV phosphodiesterase, basic and acid phosphomonoesterases, and RNase activities are present in the dark brown solution.

DEAE-CELLULOSE FRACTIONATION. A column of DEAE-cellulose ($4.9 \text{ cm}^2 \times 19.5 \text{ cm}$) is equilibrated with 0.01 M Tris buffer (pH 7.5), then washed with 2 l. of the same buffer. Half the volume of fraction IV (*i.e.*, 66.5 ml) is poured on the column at a rate of 60 ml/hr and then followed by 100 ml of 0.01 M Tris buffer (pH 7.5). A linear gradient from 0 to 0.2 M NaCl in 0.01 M Tris buffer (pH 7.5) is applied. The flow rate is 85 ml/hr, and 10-ml fractions are collected. Approximately, 85% of the activity is eluted in a sharp peak between 2.1 and

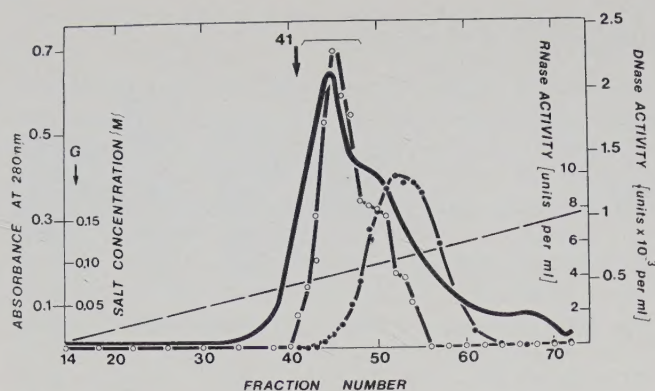


FIGURE 2: Chromatography of fraction VI on DEAE-Sephadex A-50: (—) absorbance at 280 nm (left outer scale); (●—●) RNase activity (units per ml) (right minor scale); (○—○) DNase activity (units per ml) (left outer scale); and the broken line is the molarity (left inner scale). (For details, see text.) Arrow marked G corresponds to the starting of the gradient.

3.6 resin bed volumes of effluent. The peaks of endonuclease and alkaline phosphomonoesterase activities coincide. RNase and acid phosphomonoesterase activity peaks are eluted between 2.8 and 4.2 resin bed volumes for the former and 3.6 and 5.1 for the latter. Acid phosphodiesterase activity is eluted as a broad peak between 2.4 and 4.0 resin bed volume. A second column is run with the second half of fraction IV.

Endonuclease fractions from the two columns with a specific activity greater than 200 are pooled (136 ml) and concentrated for the next step by adding 88 g of ammonium sulfate under continuous stirring. The precipitate is allowed to collect for 3 hr, recovered by centrifugation, then dissolved in a minimum amount of 0.01 M Tris buffer (pH 7.5) (fraction V: 9.5 ml, 16,850 units/ml, 55.6 mg of protein per ml). This fraction contains 50% of the activity applied to the column and can be stored at 4° for 2 weeks without any loss of activity. This step leads to an almost complete elimination of the acid phosphomonoesterase and acid phosphodiesterase and to the removal of more than 80% of the RNase.

GEL FILTRATION ON SEPHADEX G-100. A column of Sephadex G-100 (4.9 cm² × 39.5 cm) is prepared and washed with 2 l. of 0.01 M Tris buffer (pH 7.5). The same buffer is used for elution of the protein solution. An aliquot of 3.2 ml of fraction V is loaded on the column and eluted at 40 ml/hr rate. Fractions of 5 ml are collected. The elution volume of the midpoint of the alkaline phosphatase activity peak is 62 ml and that of deoxyribonuclease activity 98 ml, resulting in a complete separation of the two activities (Figure 1). The peak fractions which contain nuclease without phosphomonoesterase activity are pooled. The column is washed and used again for the two other aliquots. All the peaks containing nuclease activity are pooled. This pool is designated fraction VI (116 ml: 1120 units/ml, 0.7 mg of protein per ml); 78% of the proteins applied to the column is recovered, and a fivefold purification of the material is achieved.

DEAE-SEPHADEX CHROMATOGRAPHY. A column of DEAE-Sephadex (4.9 cm² × 30 cm) is equilibrated with 0.01 M Tris buffer (pH 7.5). Fraction VI (116 ml) is applied to the resin, then washed with 20 ml of 0.01 M Tris buffer (pH 7.5). A linear gradient (0.01–0.18 M NaCl) in 0.01 M Tris buffer (pH 7.5, total volume 500 ml) is applied. Fractions (9 ml) are collected. DNase activity appears as a main peak showing a shoulder probably due to the shrinking of the resin (Figure

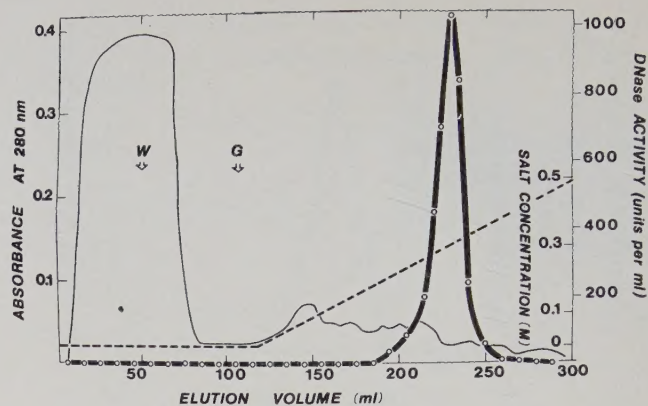


FIGURE 3: Chromatography of fraction VII on P-cellulose. Arrows marked W and G show the fraction corresponding to the washing of the column by the equilibration buffer and to the start of the gradient, respectively. (—) Absorbance at 280 nm; (○—○) endonuclease activity (right outer scale); (---) molarity of NaCl in the effluent (left inner scale).

2). RNase activity is almost completely separated from DNase. The peak fractions under brackets which contain less than 4 units per ml of RNase are pooled and adjusted to pH 5.8 by the slow addition of 0.1 N acetic acid (fraction VII: 61.5 ml, 1530 units/ml, 0.53 mg of protein per ml). When DNase completely devoid of any traces of RNase, as checked by analytical centrifugation of RNA (see Material and Methods), is needed, fraction 41 is used (see below, inhibition by polyribonucleotides).

PHOSPHOCELLULOSE CHROMATOGRAPHY. Phosphocellulose is equilibrated with 0.02 M acetate buffer (pH 5.8). A column (1.95 cm² × 12 cm) is prepared and washed with 500 ml of the same buffer. Fraction VII (61.5 ml) is applied to the resin in 35 min, then washed with 45 ml of 0.02 M acetate buffer (pH 5.8). A linear gradient (0–0.8 M NaCl) in 0.02 M acetate buffer (pH 5.8) (total volume 200 ml) is applied at a flow rate of 40 ml/hr, and 5-ml fractions are collected (Figure 3). The *Helix* DNase is eluted at 0.3 M. Fraction VIII (29 ml, 550 units/ml, and 30 μg per ml of proteins) is used, unless otherwise stated, in the studies to be reported here and in the following papers (Laval and Paoletti, 1972; Laval *et al.*, 1972²).

CRITERIA OF PURITY. Fraction VIII containing *Helix* DNase proves to be homogeneous as judged by polyacrylamide gel electrophoresis (Figure 4). Phosphomonoesterases are assayed as described in Material and Methods. Upon 6-hr incubation of 70 units of enzyme, protected by bovine serum albumin at either pH 4.3 or at pH 9.1 in the presence of Mg²⁺, there is no liberation of *p*-nitrophenol. A similar assay run on bis(*p*-nitrophenyl) phosphate rules out the presence of unspecific phosphodiesterase. Exonuclease activity is also absent, as no mononucleotides are detected by chromatography on DEAE-cellulose in 7 M urea, according to the method of Tomlinson and Tenner (1963), of an extensive digest of DNA by the *Helix* endonuclease (for details, see accompanying paper). The P-cellulose fraction VIII contains a slight ribonuclease contaminant; 55 units of DNase of this fraction liberate 10⁻³ nmole of P-RNA per min. It should be recalled that the first part of the DEAE-Sephadex fraction (Figure 2, tube number 41) is devoid of any RNase contami-

² Unpublished data.

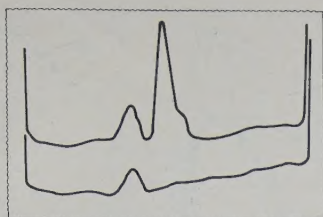


FIGURE 4: Microdensitometer tracings of polyacrylamide gel electrophoretograms of *Helix* DNase. Lower: fraction VIII (4 μ g); upper: fraction VIII (4 μ g) and pancreatic DNase (5 μ g) have been run simultaneously in order to compare the two proteins. Electrophoretic conditions are described in Materials and Methods. The origin is on the left.

nation, as checked on the ultracentrifugation assay of RNA described in Materials and Methods.

STABILITY. Upon conservation at 4° half of the activity is lost in about 2 months, but no loss of activity occurs upon conservation in 40% glycerol at -20° for 5 months (less than 5%).

Physical and Chemical Properties of the Enzyme Protein.

MOLECULAR WEIGHT. Molecular weight of *Helix* endonuclease is estimated by Sephadex G-100 gel filtration according to the procedure described by Andrews (1964). The column is successively calibrated with cytochrome *c*, soya bean trypsin inhibitor, chymotrypsin, and albumin by measuring the elution volume (V_e) of each protein in a separate run. The void volume (V_0) is the elution volume for a high molecular weight substance completely excluded from the gel particles (Dextran Blue).

In 0.05 M Tris-HCl buffer-0.1 M KCl (pH 7.5), the enzymatic activity is eluted as a single sharp symmetric peak and a molecular weight of 30,000 is calculated (Figure 5) assuming that the protein is a globular one. After zone centrifugation in a sucrose density gradient according to Martin and Ames (1961), a sedimentation coefficient of 3 ± 0.1 S is determined using an $s_{20,w}$ of 1.7 S for cytochrome *c* (Ehrenberg, 1957)

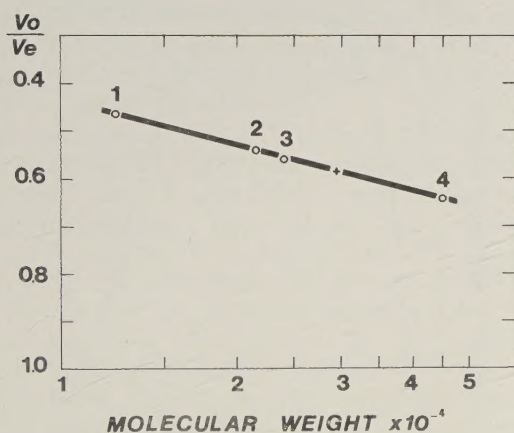


FIGURE 5: Molecular weight of endonuclease by gel filtration on Sephadex G-100. Calibration plot of V_0/V_e vs. log molecular weight of standard proteins. The ratio V_0/V_e of the proteins used as standard are plotted against logarithm of the molecular weight. V_0 void volume; V_e elution volume; 1, cytochrome *c*; 2, soya bean trypsin inhibitor; 3, chymotrypsin; 4, ovalbumin; +, 100 μ l of enzyme of the P-cellulose step is layered on the column (1 cm $\times$ 95); the enzyme activity, measured as described in Materials and Methods (General Method), is eluted as a single sharp symmetric peak. Flow rate is 12 ml/hr.

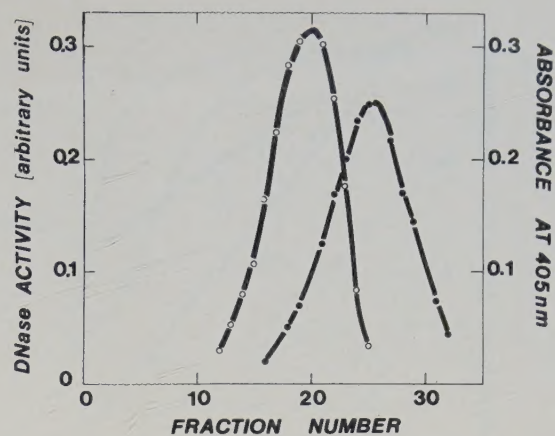


FIGURE 6: Sucrose gradient centrifugation of *Helix* DNase. A linear molarity gradient of 5-20% sucrose in 0.05 M acetate (pH 5.6) was obtained as described in Materials and Methods; 14 units of DNase (fraction VIII) and 250 μ g of cytochrome *c* are centrifuged for 16 hr at 5° at 39 krpm using a SW 39 rotor and a Spinco Model LHT ultracentrifuge. A total of 32 fractions is collected in 1 ml of 0.1 M acetate buffer containing 0.01% of bovine serum albumine, except for fractions 10, 20, 26, and 30, for which refractive index is determined prior to the addition of the buffer. (○—○) DNase activity measured at 45° (arbitrary units) (left scale); (●—●) cytochrome *c* (OD 405 nm) right scale.

(Figure 6). This value is in good agreement with the estimation of molecular weight by gel filtration.

UREA AND HEAT INACTIVATION. The rate of inactivation of *Helix* endonuclease at 37° in 10 M urea is expressed as the logarithm of the percentage of the remaining activity after incubation in 10 M urea for increasing time (Figure 7). This concentration of urea was selected after experiments which established that the enzyme is still active in the presence of

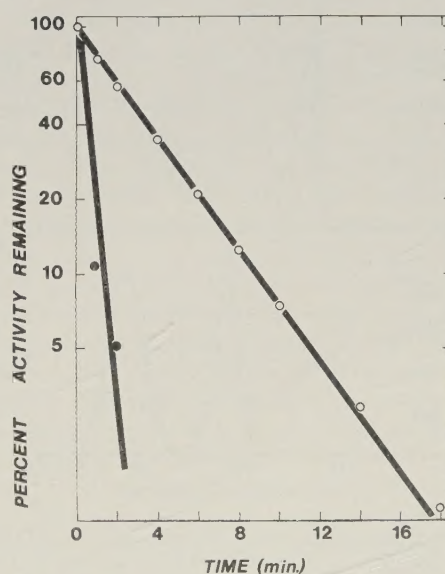


FIGURE 7: Rate of inactivation of *Helix* and splenic endonuclease by 10 M urea. At 37°, *Helix* enzyme fraction VII (0.2 ml) dialyzed against 0.1 M acetate buffer (pH 5.6) is added to 0.4 ml of 15 M urea in 0.1 M acetate (pH 5.6); 50- μ l aliquots are withdrawn and diluted one-tenth with chilled 0.1 M acetate buffer containing 0.05 M bovine serum albumin, and assayed as described under Methods. Remaining activity is determined from a plot of activity vs. incubation time. Splenic enzyme was treated under similar conditions. (○—○) *Helix* endonuclease; (●—●) splenic endonuclease.

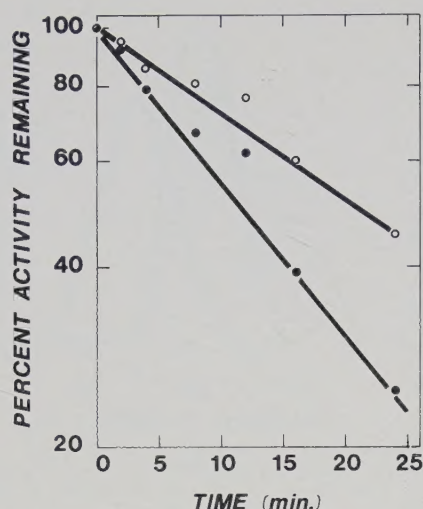


FIGURE 8: Heat inactivation of *Helix* DNase. *Helix* enzyme fraction VII is dialyzed against 0.1 M acetate buffer (pH 5.6) and heated at 60°. After different periods of time, aliquots are withdrawn, and diluted one-tenth in chilled 0.1 M acetate buffer (pH 5.6) containing 0.05% bovine serum albumin. Activity is assayed as described under Methods. Spleenic enzyme is treated under similar conditions. (○—○) *Helix* endonuclease; (●—●) spleen endonuclease.

8.0 M urea. *Helix* DNase is more resistant to urea denaturation than spleen enzyme. Half-lives of the two enzymes being respectively 3 min and 15 sec under the same experimental conditions.

The percentage of the remaining activities after incubation of *Helix* and spleen endonucleases at 60° is shown in Figure

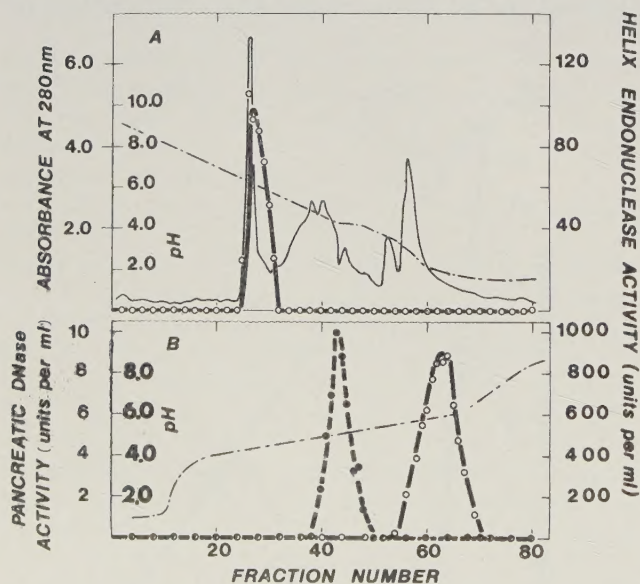


FIGURE 9: Electrofocusing of *Helix* endonuclease. A. Extract treated by dichloromethane. Fraction II is prepared for this experiment as described above (3.4 ml, 1390 units, 56 mg of proteins). Electrofocusing is done with Ampholine (pH range 3.10); voltage is 500 V for 45 min, then gradually increased to 700 V for 65 hr. Anode at the top. Recovery of DNase activity is 61%. (—) A_{280} ; (○—○) *Helix* DNase activity. B. Purified *Helix* DNase and reference protein (pancreatic DNase). 8000 units of *Helix* DNase (fraction VIII) and 30 μ g of pancreatic DNase are submitted to electrofocalization in Ampholine (pH range 4–6); voltage of 400 V, 3 mA, rising to 500 V, 2 mA in the first 2 hr. Anode at the bottom. Recovery of *Helix* DNase is 68%. (○—○) *Helix* DNase; (●—●) pancreatic DNase.

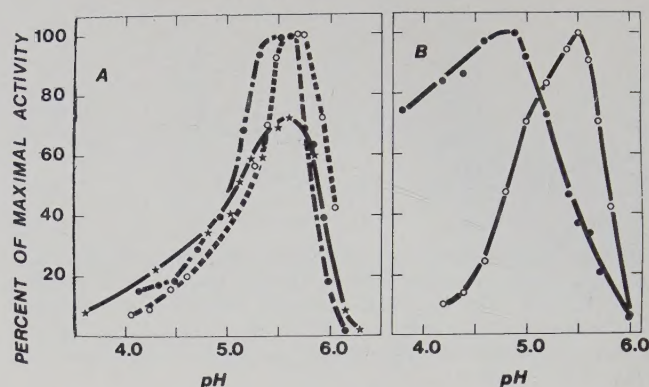


FIGURE 10: Activities of *Helix* and spleen endonucleases as a function of pH and solvent. A. *Helix* endonuclease. Citrate buffer: 0.05 M (★). Sodium acetate buffer: [acetate] 0.1 M (●) and 0.05 M (○). B. Comparison of *Helix* and spleen endonuclease activities. Ionic strength acetate buffer [Na] 0.1 M.

8. The rate of thermal inactivation is not very different for the two enzymes.

ELECTROFOCUSING EXPERIMENTS. Figure 9b shows that the isoelectric point of *Helix* DNase is 5.9 ± 0.1 . The pancreatic DNase used as a marker displays an isoelectric point of 4.95, a result in agreement with several published values obtained from other techniques (Kunitz, 1949; Polson, 1957). Another electrofocusing experiment is performed on a roughly purified extract (fraction II) in order to ascertain that the extract does not contain another acid DNase which could be lost during the purification procedure. Figure 9a shows that this is not the case, as in a large pH range (3–10) only one single symmetric peak of endonuclease activity can be characterized with an isoelectric point of 5.9 ± 0.1 .

Catalytical Properties of the Enzyme. ENVIRONMENTAL CONDITIONS. pH. The *Helix* endonuclease displays optimal activity between 5.3 and 5.6, whereas beef spleen acid DNase shows a broad peak between 3.8 and 5.0 under identical ionic

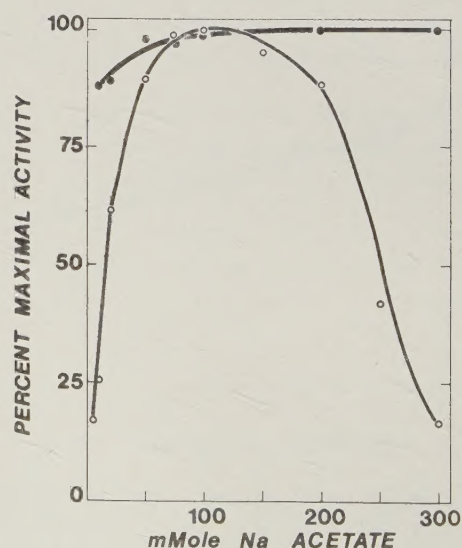


FIGURE 11: Activity of *Helix* endonuclease as a function of ionic strength. Data obtained with the spleen acid endonuclease are presented for comparison. Determination according to the general method. Solvent is acetate buffer at pH 5.6 for the *Helix* enzyme (○—○) and pH 4.8 (0.01 M EDTA) for the spleen enzyme (●—●).

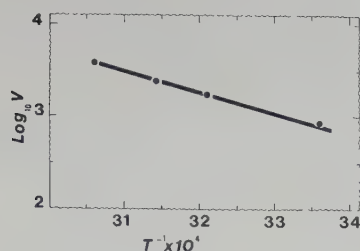


FIGURE 12: Activity of *Helix* endonuclease *vs.* temperature. The initial velocity of enzyme is determined at 25°, 37°, 45°, and 54° with the general method.

strength. With the *Helix* enzyme similar results are obtained either with acetate or citrate buffer (Figure 10).

Ionic Strength. Influence of ionic strength is critical. The protein exhibits a low catalytic activity at low and high ionic strength and is nearly fully active only in a limited range of molarity (about 0.025–0.200 M). This property is at variance with the splenic DNase (Figure 11).

Effect of Different Ions. Several ions are tested within a wide range of concentration usually from 0.5 μ mole per ml to 20 μ moles per ml. They are all inhibitory to different extents and no divalent cations are necessary for maximum activity. Dithiothreitol (0.01 M final concentration) has no effect on the enzyme activity. Mg^{2+} and SO_4^{2-} both exhibit inhibitory effects, as described also for the splenic enzyme (Cordonnier and Bernardi, 1968).

Temperature. The apparent energy of activation is 11.3 kcal. The enzyme is still fully active at 54°; it undergoes a progressive denaturation at 60° as previously described (Figure 12).

Inhibitory Effect of RNA and Synthetic Polyribonucleotides. Total rat liver RNA, poly(A), poly(I), poly(C), poly(U), and the complex poly(rA:rU) at concentrations between 1 and 20 μ g per ml show no inhibition, if assay is performed in 0.1 M acetate buffer (pH 5.6). However, at pH 5.0, the *Helix* endonuclease can be inhibited of about 50% in the presence of 2 μ g of total RNA in the incubation mixture; it should be recalled that, at pH 5.0, the activity of the enzyme is already diminished of 40% as compared to the activity at pH 5.6.

KINETICS. The K_m of *Helix* endonuclease is 1.3×10^{-5} M in 0.1 M acetate (pH 5.6) (Figure 13).

Lesca and Paoletti (1969) isolated from the liver of mouse a protein inhibitor of acid DNase; this inhibitor was shown to change the kinetics of acid DNase from a Michaelian one (without inhibitor) to a sigmoidal one (with inhibitor). We therefore investigated the kinetics of *Helix* endonuclease in the presence and in the absence of this inhibitor. For each substrate concentration the initial enzyme velocity is measured. Results are shown in Figure 13B. The kinetics in the absence of inhibitor obey Michaelis law, whereas in its presence the plot of V *vs.* (S) results in a sigmoidal-shaped curve, the inhibition being less pronounced for high substrate concentrations.

Discussion

An acid endonuclease from the hepatopancreas of a common snail *Helix aspersa* Müll. has been purified 1100-fold and obtained as a homogeneous preparation as judged by gel electrophoresis of one single species of protein of molecular weight 30,000, devoid of detectable contaminating activities

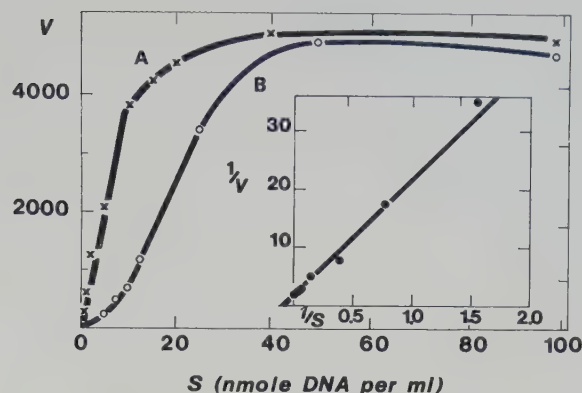


FIGURE 13: *Helix* endonuclease activity as a function of substrate concentration. A. Control solvent is 0.1 M acetate buffer (pH 5.6); temperature 37°; activities are determined with the general method ($\times$ — $\times$). Insert: Lineweaver-Burk plot of graph A. B. In the presence of the acid DNase inhibitor. Inhibitor: (1.4 U of inhibitor per ml) ($\circ$ — $\circ$).

except for traces of RNase; the ratio of DNase/RNase activity being of the order of 5×10^4 . This low level of contamination is not critical for most experiments.

Moreover, there are two ways to get rid of this contamination if needed. (a) Select a portion of the DEAE-Sephadex peak which does not contain any RNase before further processing the preparation; such a procedure results in a loss of DNase; (b) perform a complementary purification step in the electrofocusing apparatus (unpublished experiment). It should be noted that the separation of DNase from RNase by DEAE-Sephadex chromatography as described in this paper also has been efficient in our hands for beef acid spleen DNase and pancreatic DNase (unpublished results).

Our purification procedure has been elaborated in order to avoid extreme conditions of pH which were described by Kunitz (1949) for pancreatic DNase and by Bernardi and Griffe (1964) for acid splenic DNase. These latter authors emphasize the risk of a partial hydrolysis of glycosidic linkages by using low pH. Although the evidence of such linkages in *Helix* endonuclease is not established, they have been found in pancreatic DNase (Catley *et al.*, 1969) as well as in spleen DNase (Bernardi *et al.*, 1965); pH 2.5 precipitation followed by ammonium sulfate fractionation has nevertheless been attempted successfully. The recovery of the enzyme is low partly because the central parts of the chromatographic peaks displaying the highest specific activities or less contamination by other enzymes are purposely selected. The decrease of activity from fraction I to fraction VIII is not due to the disappearance of other endonuclease(s) present in crude extracts because the electrofocusing of fraction II reveals only the endonuclease presently described.

It should be noted that the enzyme displays some affinity for anionic (phosphocellulose) as well as for cationic (DEAE-Sephadex and DEAE-cellulose) chromatographic exchange resins; this holds also for other enzymes such as *E. coli* RNA polymerase (Burgess, 1969). The K_m of the *Helix* endonuclease is of the same order of magnitude as the ones measured for the splenic DNase (1.2×10^{-5} M) (Jacquemin-Sablon *et al.*, 1964) and for *E. coli* endonuclease I (2.5×10^{-5} M) (Lehman *et al.*, 1962), but well below that of the pancreatic DNase (about 5×10^{-3} M) (Le Talaer *et al.*, 1962).

No data can ascertain the monomeric or oligomeric structure of this enzyme. Consequently, any discussion on the

inhibitory effect of the spleen DNase inhibitor on *Helix* endonuclease is unwarranted; it should be recalled that the model of action at the molecular level of this inhibitor predicts a dimeric structure of the enzyme for explaining the non-Michaelian kinetics (Lesca and Paoletti, 1969).

Other noticeable characteristics of this enzyme are: narrowness of the pH range of activity (50% of maximum activity at pH 5.0 and 5.9), reduction of activity at both low and high ionic strength, and resistance to the denaturation by urea.

Acid DNase activities present in different species are thought to be associated with proteins very similar with respect to their chromatographic and enzymatic properties and sedimentation coefficients (Cordonnier and Bernardi, 1968).

In spite of sharing some of those common properties (molecular weight, chromatography on hydroxylapatite (unpublished data), and reduced activity toward denatured DNA (Laval *et al.*, 1966; Laval and Paoletti, 1972)) *Helix* endonuclease is definitely an enzyme different from the former ones, especially from the hog splenic acid DNase, which is the most investigated in this series, according to the following criteria: (a) isoelectric point, 5.9 for *Helix* enzyme and 10.2 for the hog spleen enzyme (Bernardi, 1968), although different techniques have been used; (b) pH dependency; (c) ionic strength dependency; (d) chromatographic behavior, in our hands, the *Helix* DNase is recovered in the breakthrough peak on CM-Sephadex (0.05 M phosphate buffer, pH 6.8), whereas the spleen enzyme is retained in the same conditions, and (e) the specificity toward the base sequences which shows striking differences (Laval *et al.*, 1972).² Moreover, the hepatopancreas of *Helix* does not contain any endonuclease which could be similar to the splenic enzyme or to the endonuclease which is present in another gasteropode, the clam *Mercenaria* (Cordonnier and Bernardi, 1968). Studies on the four endonucleases from another mollusc, *Octopus vulgaris*, are too preliminary to decide to which group these enzymes belong (Antonoglou and Georgatsos, 1968).

Acknowledgments

The authors wish to thank Dr. G. Bernardi for critical interest, Dr. F. Laval for help during the course of this work, Dr. J-B. Le Pecq and Dr. A. Jacquemin-Sablon for stimulating discussions, Dr. R. P. Dechambre for taxonomic identification of the *Helix*, and Miss C. Delaporte for her excellent technical assistance.

References

- Andrews, P. (1964), *Biochem. J.* 91, 1964.
- Antonoglou, O., and Georgatsos, J. G. (1968), *Arch. Biochem. Biophys.* 127, 813.
- Bernardi, G. (1968), *Advan. Enzymol.* 31, 1.
- Bernardi, G., Appella, E., and Zito, R. (1965), *Biochemistry* 4, 1725.
- Bernardi, G., and Grifffé, M. (1964), *Biochemistry* 3, 1419.
- Bernardi, G., Griffe, M., and Appella, E. (1963), *Nature (London)* 198, 186.
- Burgess, R. R. (1969), *J. Biol. Chem.* 244, 6160.
- Catley, B. J., Moore, S., and Stein, W. H. (1969), *J. Biol. Chem.* 244, 933.
- Chersi, A., Bernardi, A., and Bernardi, G. (1966), *Biochim. Biophys. Acta* 129, 12.
- Cordonnier, C., and Bernardi, G. (1968), *Can. J. Biochem.* 46, 989.
- Davis, J. J. (1964), *Ann. N. Y. Acad. Sci.* 121, 404.
- Defrance, P., and de Lesdain, N. (1962), *Pathol. Biol.* 10, 153.
- Ehrenberg, A. (1957), *Acta Chim. Scand.* 11, 1257.
- Hiatt, H. H. (1962), *J. Mol. Biol.* 5, 217.
- Jacquemin-Sablon, A., Laval, J., Le Talaer, J. Y., Le Pecq, J. B., and Paoletti, C. (1964), *C. R. Acad. Sci.*, 259, 2551.
- Kelly, T. J., and Smith, H. O. (1970), *J. Mol. Biol.* 51, 393.
- Kunitz, M. (1949), *J. Gen. Physiol.* 33, 349.
- Laval, J., Laval, F., and Paoletti, C. (1966), *C. R. Acad. Sci.* 262, 2093.
- Laval, F., Malaise, E., and Laval, J. (1970), *Exp. Cell Res.* 63, 69.
- Laval, J., and Paoletti, C. (1972), *Biochemistry* 11, 3604.
- Le Pecq, J. B., and Paoletti, C. (1966), *Anal. Biochem.* 17, 100.
- Lesca, P., and Paoletti, C. (1969), *Proc. Nat. Acad. Sci. U. S.* 64, 913.
- Le Talaer, J. Y., Festy, B., Le Pecq, J. B., Paoletti, C. (1962), *Nature (London)* 194, 1176.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem.* 193, 265.
- Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem.* 236, 1372.
- Ornstein, L. (1964), *Ann. N. Y. Acad. Sci.* 121, 321.
- Paul, A. V., and Lehman, I. R. (1966), *J. Biol. Chem.* 241, 3441.
- Polson, J. (1957), *Biochim. Biophys. Acta* 22, 67.
- Privat de Garilhe, M., and Laskowsky, M. (1955), *Biochim. Biophys. Acta* 18, 370.
- Schneider, N. C., and Hogeboom, G. (1952), *J. Biol. Chem.* 198, 155.
- Sih, C. J., Laval, J., and Rahim, C. (1963), *J. Biol. Chem.* 238, 566.
- Taborsky, G. (1959), *J. Biol. Chem.* 234, 2652.
- Tomlinson, R. V., and Tener, G. M. (1963), *Biochemistry* 2, 697.

Mechanism of Deoxyribonucleic Acid Degradation by an Acid Deoxyribonuclease from the Snail *Helix aspersa* Müll.[†]

Jacques Laval* and Claude Paoletti

ABSTRACT: The acid deoxyribonuclease extracted from the hepatopancreas of the snail (*Helix aspersa* Müll.) is specific for deoxypolymers. Glycosylated (T4 DNA) and nonglycosylated DNA, as well as poly[d(A-T)]·poly[d(A-T)], are hydrolyzed, whereas poly[d(G-C)] is not. The enzyme is an endonuclease since it opens covalently closed PM2 circular molecules of DNA. The initial velocity of the enzyme is 30 times higher when acting on native DNA than on denatured DNA. The denatured DNA is not an inhibitor of the enzyme. Circular molecules of PM2 untwisted by the sequential use of

pancreatic DNase and *Escherichia coli* polynucleotide ligase are hydrolyzed at the same rate as twisted natural ones. The enzyme hydrolyzes DNA according to both diplotomic (single hit) and haplotomic (double hit) mechanisms. It yields products bearing the monoesterified phosphate attached to position 3'. The oligonucleotides produced after an exhaustive digestion (average chain length 6.4) contains barely detectable mono- and dinucleotides and very few trinucleotides. Their average length ranges between 4 and 10.

The hepatopancreas of the common snail (*Helix aspersa* Müll.) contains an acid DNase. In the previous paper (Laval and Paoletti, 1972) we have described its purification, established its main physical and chemical properties, and compared it with another acid DNase: the beef spleen endonuclease. Availability of this enzyme in a pure form, free of contaminating enzymatic activities such as exonuclease, phosphatase, and unspecific phosphodiesterase, enables us to study its specificity, according to the origin and the secondary and tertiary structures of the DNA used as substrate, and its initial kinetics of action (haplotomic *vs.* diplotomic mode of hydrolysis). The nature of oligonucleotides produced after digestion of DNA, *i.e.*, their average length and the localization of the phosphate terminal were also investigated.

Materials and Methods¹

Urea is a Merck product and is recrystallized from ethanol prior to use. DEAE-cellulose (Selectacel, type 20, capacity 1 mequiv/g) is purchased from Schleicher and Schuell, Keene,

N. Y.). Sucrose is an Analar product, and Tris is from Sigma, St. Louis, Mo. Solutions and buffers are made with glass-distilled water and are sterilized prior to their utilization. Dialysis tubings are boiled twice in a 10% sodium carbonate-0.01 M EDTA solution, washed with water until a neutral pH is reached, and sterilized. Dialysis tubing is handled with sterile surgical gloves.

Enzymes. *Helix* endonuclease (fraction VIII) and beef spleen acid DNase have been described in the preceding paper (Laval and Paoletti, 1972). Spleen phosphodiesterase, pancreatic DNase (3-times recrystallized), and snake venom phosphodiesterase are purchased from Worthington Biochemical Corp., Freehold, N. J. The preparation of polynucleotide ligase from *Escherichia coli* is already described (Paoletti *et al.*, 1971).

Nucleic Acids. Calf thymus DNA (CTS) is prepared as described by Signer and Schwander (1949). The molar extinction coefficient, measured at 260 nm as moles of nucleotide per liter, ϵ (P), is 6900. Sedimentation coefficient is 24 S.

The preparation of ³H-labeled *E. coli* DNA has been described already (Laval *et al.*, 1970) (Laval and Paoletti, 1972). ¹⁴C-labeled *E. coli* is prepared by the same method as used to prepare ³H-labeled *E. coli*. Its specific activity is 3.2×10^6 cpm/ μ mole. When using denatured DNA, ¹⁴C-labeled DNA is denatured just before the experiment by heating for 10 min in a boiling water bath (15 μ g/ml in 0.005 M NaCl then chilling in an ice bath. Hyperchromicity at 260 nm is 25%. By reference to the C_{0t} curve established by Britten and Kohne (1968) for *E. coli* DNA, one may assume that after 90 min of incubation there is less than 1% renatured DNA in our conditions.

DNA from [³H]thymidine-labeled T7 phage is extracted according to Richardson *et al.* (1964), except for the first step of purification of phages which is performed by phase partition (Philipson *et al.*, 1960). The specific activity of T7 DNA is 2.4×10^6 cpm/ μ mole; 74% of this DNA sediments in an alkaline sucrose gradient as an homogeneous component during zone sedimentation.

Poly[d(A-T)]·poly[d(A-T)] and poly[d(G·C)] are purchased from Miles Research laboratories. DNA from bacteriophage PM2 is prepared as described by Paoletti *et al.* (1971). Its

[†] From the Laboratoire de Biochimie et Enzymologie, Institut Gustave-Roussy, 94-Villejuif, France. Received May 15, 1972. This work is a contribution from the Laboratoire de Pharmacologie moléculaire associé au Centre National de la Recherche scientifique (CNRS). This work has been aided by the Institut National de la Santé et de la Recherche Médicale (INSERM), Paris, France, Ligue Nationale contre le Cancer, Paris and Commissariat à l'Energie Atomique, 91 Gif-sur-Yvette, France. A preliminary account of this work was presented at the Sixth Meeting of the Federation of European Biochemical Societies Madrid, April 1969.

¹ Abbreviations used are: solution A, standard solution made up of ethidium bromide (1 μ g/ml) dissolved in 0.01 M Tris-HCl (pH 7.8)–0.02 M NaCl–0.005 M EDTA; IF, fluorescence intensity; Δ IF, variation of IF expressed as units on the scale of the PMQ II Zeiss spectrometer equipped with ZF M 4 fluorimeter. We define a single-strand break (ss break) as the hydrolysis of a phosphodiester linkage on one strand only. When two ss breaks on each individual strand either face each other, or are sufficiently close they generate a double-strand break (ds break), *i.e.*, a rupture of the duplex. The maximal distance between the breaks on opposite strands which allows the duplex to be stable depends on the environmental conditions, temperature, and ionic strength (Freifelder and Trumbo, 1969). When an enzyme delivers only ss breaks, its mechanism of action is defined as haplotomic, when it delivers ds break, this mechanism is defined as diplotomic (Bernardi, 1968).

superhelix density is 0.047 as determined by Revet *et al.* (1971) and Paoletti (unpublished data); untwisted PM2 DNA is prepared by the simultaneous action of pancreatic DNase and *E. coli* ligase as described by Paoletti *et al.* (1971).

Assays

Assays of *Helix* DNase. Enzyme activity is assayed in most cases as described in the preceding paper (Laval and Paoletti, 1972). The assay is also performed according to Kunitz (1949). Hyperchromic shift is followed in a jacketed silica cell using a Zeiss PMQII spectrophotometer.

Viscosity Measurements. For deciding between an haplotomic and a diplotomic mode of action of the enzyme, we follow the changes of viscosity of an enzyme DNA mixture using a viscosimeter Lecomte du Nouy "Couette" type, at shear gradient 15 sec^{-1} , temperature 25° . Kinetic data obtained by following the intrinsic specific viscosity during enzymatic degradation of the DNA are treated according to Schumaker *et al.* (1956). The apparent number of strands, n , is obtained from the slope of $\log(1 - R)/R$ vs. $\log t$. R is equal to M_t/M_0 or η_t/η_0 , where M_t , M_0 , η_t , and η_0 are, respectively, average molecular weight and the specific viscosity at digestion time t and zero time.

Fluorescence Measurements. The rate at which the first break is induced on each DNA molecule is determined by establishing the kinetics of the opening of closed circular PM2 DNA. For this purpose, we choose the fluorometric method described by Paoletti *et al.* (1971) based on the differential affinity of ethidium bromide for open and closed DNA circular molecules.

Assay of Exonucleases. The liberation of uranyl perchloric soluble material is used as a measurement of enzyme activity: To $400 \mu\text{g}$ of native or *Helix* nuclease treated DNA in $500 \mu\text{l}$ of the proper buffer is added 1 ml of a cold aqueous solution of 2.5% HClO_4 and 0.25% uranyl acetate. After 5 min at 0° , the precipitate is centrifuged 10 min at $12,000g$. Absorbance of the supernatant is read at 260 nm against an appropriate blank.

Zone Sedimentation of DNA on Sucrose Density Gradients. Sedimentation is carried out in linear concentration gradients of sucrose ($5\text{--}20\%$) according to Richardson (1966). Neutral conditions are 0.01 M Tris-HCl- 1 M NaCl- 0.001 M EDTA (pH 8.0). Alkaline conditions are 0.11 M NaOH- 0.89 M NaCl- 0.001 M EDTA (pH 12.0). Gradients are prepared by use of a gradient maker device and a polystatic pump (Büchler, Fort Lee, N. Y.).

The sample of T7 [^3H]DNA ($0.64 \mu\text{g}$ in 0.1 ml) is layered on 4.2 ml of sucrose. In neutral conditions, DNA is centrifuged for 165 min at $35,000 \text{ rpm}$ at 4° in the rotor SW 39 in a Spinco Model L2-65 centrifuge. Fractions (2 drops) are collected on Whatman fiber paper GF/C, dried, and counted. Recovery of radioactive material was better than 90% . In alkaline conditions, DNA is centrifuged 120 min at $52,000 \text{ rpm}$ in a SW 65 rotor.

Both under neutral and alkaline conditions the fraction of DNA sedimenting in the undegraded form, as well as in the degraded one, is established by measuring the relative weight of the area cut off under the corresponding peak.

Assuming that the breaks are randomly distributed among all the molecules, the Poisson's law applies: $p = e^{-m}$ where p is the fraction of DNA still sedimenting in an undegraded form and m the average number of ds break per DNA molecule (in neutral conditions) or the average number of ss break per strand (in alkaline conditions).

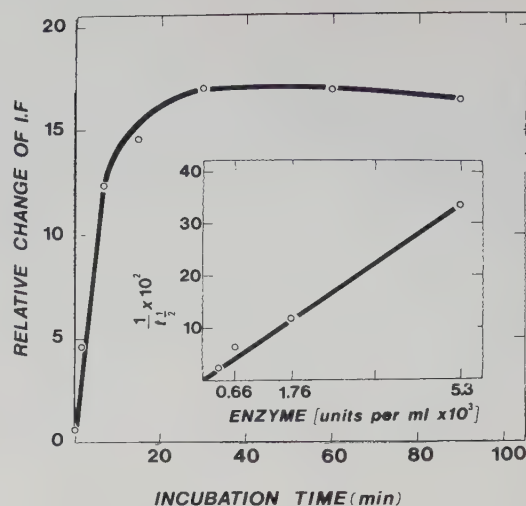


FIGURE 1: Fluorometric determination of the hydrolytic rate of *Helix* endonuclease. *Helix* DNase (0.0033 unit) in $5 \mu\text{l}$ of 0.1% ovalbumin is added to PM2 DNA in $420 \mu\text{l}$ of 0.1 M Na acetate buffer (pH 5.6) (DNA concentration: $9.91 \mu\text{g/ml}$). After different times at 37° , $40 \mu\text{l}$ of the mixture is withdrawn and added to $800 \mu\text{l}$ of solution A. For fluorometric determinations, see Materials and Methods. Insert: proportionality between the hydrolytic rate and the enzyme concentration. An experiment similar to that described above is run PM2 DNA concentration is $1.60 \mu\text{g/ml}$. For definition of $t_{1/2}$, see text. Increasing amounts of *Helix* DNase are added.

Size Determination of Oligonucleotides Produced by an Extensive Hydrolysis of DNA. The oligonucleotides are separated on a DEAE-cellulose-urea column according to Tomlinson and Tener (1963). The ultraviolet transmission of the effluent is continuously monitored at 254 nm using a LKB recorder (Uppsala, Sweden). The optical density of the fractions is measured in a Zeiss PMQ II spectrophotometer. The experimental chromatographic pattern is analyzed by the Dupont de Nemours Curve Resolver 310 which computes the proportion of each isoplith. The length of each separated isoplith is established from (i) the molarity of the elution from the column and (ii) the ratio of phosphatase-sensitive phosphorus to total phosphorus. Phosphorus determination is carried according to Martin and Doty (1949) as modified by Carrara and Bernardi (1968).

Results

Hydrolysis of DNA of Different Sources. Calf thymus DNA, T4 DNA which bears glycosylated hydroxymethylcytosine nucleotides, and poly[d(A-T)]·poly[d(A-T)] are all found to be equally good substrates (Kunitz assay) for the enzyme. Poly(dG)·poly(dC) does not show any hyperchromic effect. After sedimentation through an alkaline gradient (pH 12.5), the patterns of endonuclease-treated and nontreated poly(dG)·poly(dC) are similar. Therefore, no single-strand breaks are revealed. This result rules out a limited attack of this polymer by *Helix* nuclease. Its significance will be discussed in the third paper of this series (Laval *et al.*, 1972)² which is relative to the bases specificity of this enzyme which is unable to hydrolyze a dpCpX phosphodiester sequence.

Helix DNase is able to perform endonucleolytic breaks as shown by two different experiments. (i) This enzyme is able to open up covalently closed PM2 DNA circular molecules (Figure 1). This opening, which expresses the initial breaking events, can be followed by ethidium bromide fluorimetry

² In preparation.

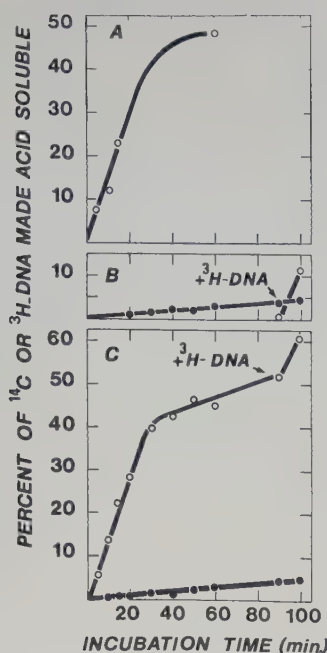


FIGURE 2: Activity of *Helix* DNase, according to secondary structure of DNA. Three series of reaction mixtures are set up, containing either ^3H labeled native or ^{14}C -labeled heat denatured or both ^3H -labeled native and ^{14}C -labeled *E. coli* denatured DNA, as described in Materials and Methods, except that incubation temperature is 49° . The mixtures were preincubated at 49° for 3 min prior to addition of 0.095 unit of enzyme per assay. (A) ^3H -labeled native DNA. (B) ^{14}C -labeled heat-denatured DNA; + ^3H -DNA indicates the addition of 15.8 nmoles of native ^3H -DNA in a volume of 10 μl . (C) Mixture of ^3H -labeled native and ^{14}C -labeled denatured DNA. + ^3H -DNA: *vide supra* B. (○—○) Per cent of total ^3H -DNA rendered acid soluble. (●—●) Per cent of total ^{14}C -DNA rendered acid soluble.

which is based on the measurement of $t_{1/2}$, time at which the increase of the fluorescence intensity due to the opening of the DNA circles is half the maximum increase when all the circles have been opened. There is an average of 0.69 opening event per PM2 circular molecule at $t_{1/2} \cdot t_{1/2}$ is reciprocal to the hydrolytic rate (Paoletti *et al.*, 1971).

In our experimental conditions [DNA concentration, 9.9 $\mu\text{g}/\text{ml}$; temperature, 37° ; solvent, 0.1 M acetate (pH 5.6)] the opening rate, as followed by fluorometry, is proportional to the enzyme concentration, in the range used for this work (Figure 1, insert).

Each unit of enzyme activity (1 nmole of DNA made acid soluble per minute at 37°) delivers 415 hits per PM2 DNA molecule per minute or 69 hits per 1×10^6 molecular weight of DNA and per minute.

(ii) Few breaks only reduce the molecular weight of T7 DNA as judged by zone centrifugation in neutral as well as in alkaline conditions (see below). This observation implies that the breakage is an endonucleolytic one. Moreover, during the action of the enzyme there is no increase of radioactivity detected at the meniscus (see below, Figure 4). Therefore, during the initial stages of degradation there is no detectable liberation of small oligonucleotides; hence the enzyme preparation is not contaminated by an exonuclease, and the endonuclease molecule is devoid of detectable exonucleolytic activity.

Effect of the Secondary Structure of DNA on Enzymatic Rate. Native and heat-denatured *E. coli* labeled DNA are compared as substrates for *Helix* endonuclease either alone or as a mixture. At 49° , the initial velocity of the attack is 30

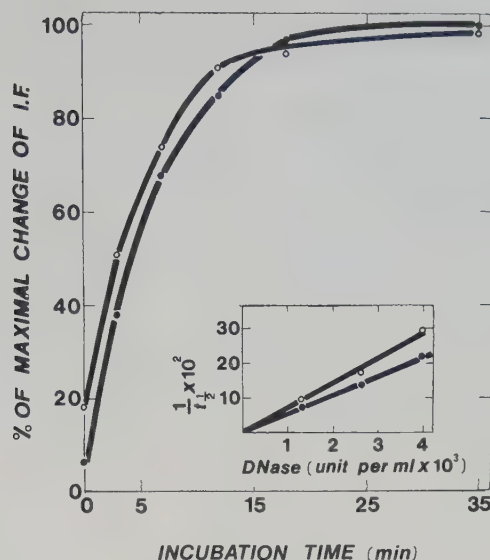


FIGURE 3: Comparative rate of opening of twisted and detwisted covalently closed circular PM2 DNA by *Helix* endonuclease. *Helix* DNase (0.004 unit) in 5 μl of 0.1% ovalbumin is added to PM2 DNA in 980 μl of 0.1 M Na acetate buffer (pH 5.6). DNA concentration for twisted closed DNA: 4.15 $\mu\text{g}/\text{ml}$, for detwisted closed DNA 5.20 $\mu\text{g}/\text{ml}$. After different times at 37° , 150 μl of each mixture is withdrawn and added to 800 μl of solution A. For fluorometric determinations, see Materials and Methods. Twisted DNA (●—●) IF before addition of enzyme, 65.4, ΔIF , 16.2; detwisted DNA (○—○) IF before addition of enzyme, 64.2, ΔIF , 33.8. IF of solution A alone is 17.0. Insert: rate of opening *vs.* enzyme concentration. Same experimental conditions as before.

times more rapid for the native form than for denatured DNA (Figure 1). The digestion of native DNA proceeds rapidly until about 45% of the substrate is rendered acid soluble and then slows down suddenly. An explanation for the suddenness of this slowing down when only a part of the DNA has been hydrolyzed could be provided by a specificity for an organized secondary structure. Another explanation would assume that denatured DNA and/or the melted products of the enzyme action are inhibitors of the enzyme. This latter explanation is ruled out by experiments shown on Figure 2. The rate of attack of DNA does not depend on the addition in the reaction medium of denatured DNA, or of reaction products of low molecular weight (Figure 2C). However, although these products do not inhibit DNA hydrolysis, they might still competitively inhibit the reaction at its end.

The results obtained at 37° support the hypothesis according to which the enzyme is specific for a duplex structure: the slowing of the rate of enzyme action occurs when more than 70% of the substrate is rendered acid soluble, instead of 45% at 49° (Figure 2A). Such a result could be expected since the double-stranded structure of comparable size is more stable at 37° than at 49° in our conditions. In each of these experiments, it has been verified that no partial denaturation of the enzyme occurs during the reaction: no loss of activity is observed when native DNA is added after 90 min of enzyme action at 49° on either native DNA (Figure 2C) or on denatured DNA (Figure 2B, C).

Effect of Supercoiling of Circular DNA on Enzyme Activity. It has been demonstrated (Dean and Lebowitz, 1971; unpublished data from our laboratory; Paoletti and Le Pecq, 1971) that supercoiling to which a positive free energy is associated causes alterations in the double-helical structure of native closed circular DNA. Since from the above

TABLE I: Average Number of Double-Strand Breaks and Single-Strand Breaks per Molecule of T7 DNA after a Limited Hydrolysis by *Helix* DNase.

Hydrolysis Time (min)	Sedimentation in Neutral Sucrose ^a		Sedimentation in Alkaline Sucrose ^a		
	32.0S Native DNA/Total DNA ^b	Average Number of ds Breaks per T7 DNA Molecule ^c	37.2S DNA/Total DNA ^b	Average Number of ss Breaks per T7 DNA Strand ^c	Ratio ds Breaks/ss Breaks per T7 DNA Molecule
0	1	0	0.74	0.3	
10	0.64	0.4	0.28	1.3	0.15
20	0.15	1.9	0.08	2.5	0.38

^a Data taken from T7 DNA zone sedimentation in Figure 4.

^b Total DNA is the DNA sedimenting at the position of native T7 marker (32S) or denatured T7 marker (37.2S) (Studier, 1965) plus the DNA sedimenting in the degraded form.

^c For the calculation method, see Materials and Methods as well as text.

data *Helix* endonuclease recognizes the secondary structure of DNA, it was interesting to determine whether the enzyme reacts in the same way toward twisted natural PM2 circular DNA and detwisted DNA (allomorphic structures), prepared by the removal of the supercoils after opening up and closing the circles through the joint action of pancreatic DNase and *E. coli* polynucleotide ligase. An answer to this question is provided by the data of Figure 3. *Helix* endonuclease induces a number of breaks per minute and per molecule of PM2 closed circular DNA, which does not depend on the twisting of the DNA, in the limits of sensitivity of the fluorometric method used here.

Predominant Diplotomic Mode of Action of *Helix* Nuclease. If an endonuclease produces only ss breaks on DNA it will not modify its average molecular weight during the initial phase of its action whereas it will decrease the average molecular weight of its strands, measured after an alkaline denaturation.

In contrast, if an endonuclease produces only ds breaks on DNA, the average molecular weight of the duplex DNA will decrease as soon as the enzymatic attack is initiated; its value will parallel that of the separated strand. In the first case (ss breaks), the ratio between ds breaks and ss breaks per molecule will be initially 1 per 200 to reduce the molecular weight to half as shown by Thomas (1956) and Freifelder and Trumbo (1970) in environmental conditions similar to those used here. In the second case (ds breaks) it should be 1 per 2, i.e., 0.5.

The number of ss breaks and ds breaks is evaluated by zone sedimentation as described in Material and Methods. Table I gives the ratio of ds breaks to ss breaks per molecule during the initial phase of attack of T7 DNA by *Helix* nuclease. For a very small average number of ss breaks per strand (about 1), the ratio of ds breaks to ss breaks per molecule is already high (0.15).

When the average number of ss breaks per strand is doubled, the ratio of ds breaks to ss breaks per molecule (0.38) is al-

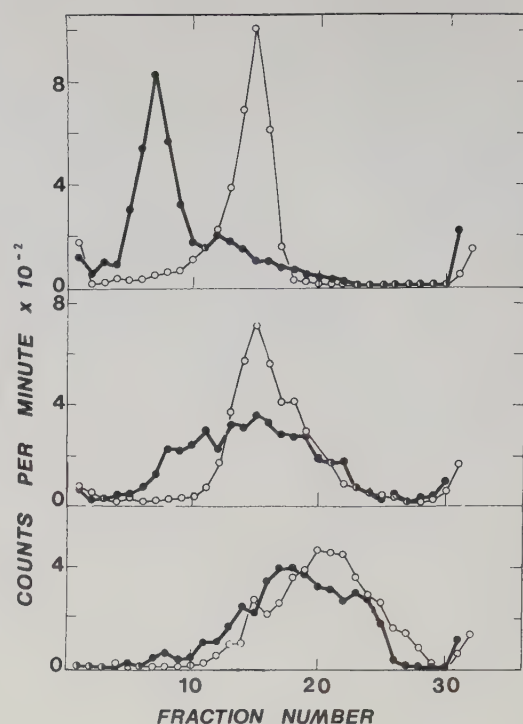


FIGURE 4: Zone sedimentation of T7 [³H]DNA degraded by *Helix* endonuclease. Incubation mixture contains 6 μ g/ml of boiled pancreatic RNase, 5.32 μ g of T7 [³H]DNA in 0.05 M acetate buffer (pH 5.6), and 0.0017 unit of *Helix* DNase; final volume 800 μ l. After 0, 10, and 20 min at 37°, 250 μ l is withdrawn, poured over 10 μ l of 1 M Tris to stop the enzymatic reaction, and kept at 0°. 0.1 ml is layered either on an alkaline or on a neutral sucrose gradient, radioactivity is determined on fractions after centrifugation as described in Materials and Methods. For the sake of simplicity, neutral and alkaline sucrose gradients are superimposed. (●—●) Alkaline gradient, (○—○) neutral gradient.

ready nearly equal to the theoretical ratio for diplotomic enzyme (0.5). Consequently, *Helix* endonuclease acts as a diplotomic enzyme delivering breaks facing each other or very close on opposite strand. However, at least during the initial stage of DNA attack, some ss breaks are produced, therefore, both haplotomic and diplotomic mechanisms are at work, the latter being widely predominant. For instance, for an average delivery of 5 ss breaks per molecule, about 80% of these breaks are located in such a position as to form ds breaks. Such a phenomenon has also been reported for several other nucleases (Bernardi, 1968).

The kinetics of DNA degradation by *Helix* DNase followed by viscosity reflects these findings.

When the kinetic data related to the changes of viscosity of DNA under the *Helix* nuclease action are treated according to Schumaker *et al.* (1956) allowing the determination of the apparent number of strand n , n is found to be 1.3 for *Helix* DNase.

Control experiments establish that in identical conditions hog spleen DNase (predominantly diplotomic) has an n value of 0.9 and pancreatic DNase (haplotomic) of 1.5, similar to the finding of Bernardi (1968).

Localization of the Phosphomonoester and Hydroxy End Groups on the Oligonucleotides Provided by *Helix* Nuclease. (a) In order to ascertain if the *Helix* nuclease yields oligonucleotides bearing 3'-P or 5'-P end groups, advantage has been taken of the specificity of the spleen and venom exonucleases. Spleen exonuclease initiates attack at free 5'-OH

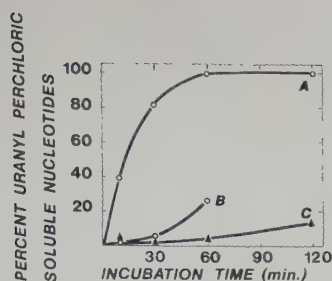


FIGURE 5: Action of spleen and venom exonucleases on DNA nicked by *Helix* nuclease. Thymus DNA (CTS) (1.375 mg) in 0.05 M sodium acetate buffer (pH 5.6) is treated with 14 units of *Helix* endonuclease in 1.5 ml for 25 min, then boiled for 10 min to destroy the enzyme activity (15.6% of DNA is rendered perchloric acid soluble while there is no uranyl perchloric acid soluble material as described in Materials and Methods). This partially hydrolyzed DNA (HP-DNA), is then used for the following experiments. Curve A: 400 μ g of HP-DNA, 8 μ moles of acetic acid, and 2 units of spleen phosphodiesterase (SPH). Final volume 500 μ l; pH 5.0. Curve B: HP-DNA is replaced by native DNA, other conditions are the same for curve A. Curve C: 400 μ g of HP-DNA, 2 μ moles of $MgCl_2$, 24 μ moles of Tris base, and 20 μ g of venom phosphodiesterase (VPH). Final volume, 500 μ l; pH 9.0. Aliquots (50 μ l) are withdrawn at various times and mixed with 150 μ l of carrier DNA (1 mg/ml) and 200 μ l of uranyl. Perchloric acid soluble material is determined as described in Materials and Methods. All experiments are run at 37°.

and liberates 3'-P mononucleotides. It does not attack polynucleotides with a 5'-phosphomonoester end group. The snake venom initiates hydrolysis at the 3'-OH end, yielding 5'-P mononucleotides. It does not attack polynucleotides with a 5'-phosphomonoester end group.

Figure 5 shows that a limited digest of calf thymus DNA by *Helix* nuclease is a good substrate for spleen exonuclease (curve A), while it is not for venom exonuclease (curve C). The slow and delayed appearance of mononucleotides upon action of the venom enzyme probably reflects its contamination by an endonuclease as described by Laskowski (1967). The same explanation [*i.e.*, presence of traces of the acid endonuclease, described by Bernardi and Grifffé (1964) in the exonuclease preparation] holds for the delayed effect of spleen exonuclease on native DNA.

These experiments demonstrate that the oligonucleotides produced by the *Helix* nuclease bear 3'-phosphomonoester end groups.

(b) The same conclusion is reached by an experiment with polynucleotide kinase which has been shown to have an absolute requirement for 5'-OH ends (Richardson, 1965). Upon incubation of DNA with *Helix* DNase, oligonucleotides appear which can be phosphorylated by polynucleotide kinase [see paper III of this series (Laval *et al.*, 1972)²]. Advantage has been taken of this property to identify the 5'-OH-terminal nucleotide in *Helix* DNase digests.

Determination of the Length of the Oligonucleotides Liberated after an Exhaustive Degradation of DNA by *Helix* Endonuclease. The average length of the oligonucleotides of an exhaustive digest is 6.4 as determined by the ratio of phosphatase-sensitive phosphorus to total phosphorus.

When these oligonucleotides are fractionated according to their length on a DEAE-cellulose-urea column (Figure 6) the relative proportion of each isoplith can be estimated (Table II). No oligonucleotide of chain length higher than 12 is present. No mononucleotides and very few, if any, dinucleotides and trinucleotides are recovered, even if the column is purposely overloaded. These data confirm that the enzyme

TABLE II: Chromatography of *Helix* Digest on DEAE-Cellulose-Urea Column.^a

Proportion of Each Isoplith (Per Cent of Total Absorbance)						
<IV	IV	V	VI	VII	VIII	>VIII
1.0	5.5	16.5	23.7	19.2	13.4	20.7

^a Incubation mixture of the digest contains 500 OD₂₆₀ of DNA (CTS) and 1.670 units of *Helix* DNase in 0.05 M Na acetate buffer (pH 5.6), and 2 drops of toluene; final volume: 15 ml. Incubation: 37° for 19 hr. Isopliths are fractionated on a DEAE-cellulose-urea column (see Figure 6). IV to VIII refer to tetra- to octanucleotides.

preparation is devoid of an exonucleolytic activity either embodied in the endonucleolytic protein or present as a contaminant.

Discussion

Helix nuclease is an endonuclease since it is able to open covalently closed circular DNA molecules. It is free of detectable amounts of contaminating true exonuclease *sensu stricto* since it does not yield mono- or dinucleotides. Moreover, it yields only one band in polyacrylamide gel electrophoresis.

The *Helix* enzyme can only accommodate oligonucleotides at least eight monomers long, since the heptanucleotides and smaller oligonucleotides would yield either mono-, di-, or trinucleotides which are not found after isosteach separation. An upper limit of the length of the oligonucleotides completely sensitive to the enzyme is represented by decanucleotides. These are barely detectable. Thus, ability of the oligonucleotides to be attacked by the *Helix* enzyme is highly length dependent, in the range 8–10 nucleotides. Such a dependency is also found for other depolymerizing enzymes, such as lysozyme which degrades pentamers and hexamers 500 and 3750 times faster than tetramers (Jollès, 1964).

One cannot rule out the possibility that such a narrow range of average chain length could merely be the consequence of an accumulation of sequences of bases resistant to hydrolysis, because the enzyme is highly specific for specific base sequences as demonstrated in the accompanying paper (Laval *et al.*, 1972).² However, the published results (Lehman *et al.*, 1962) on the mode of action of endonuclease I from *E. coli* does not favor this hypothesis. This nuclease has a molecular weight close to that of the *Helix* enzyme and gives also mainly heptanucleotides after exhaustive digestion of DNA in spite of absence of known base sequences specificity.

Another explanation for the relatively precise and short average length of the oligonucleotides produced by *Helix* enzyme lies on the assumption that their size depends on the size of the enzymatic protein. The mean radius of a protein molecule having a molecular weight of 30,000, such as *Helix* DNase, should be in the range of 1.5–4 nm depending on its shape. Accordingly, it should accommodate a zone 5–12 base pairs long on the duplex DNA, which is in good agreement with the actual average length of oligonucleotides resulting from an exhaustive action of the *Helix* DNase (6.4 nucleotides). Such an hypothesis implies that breaks or some truly endonucleolytic breaks are accompanied by the adjacent removal of one or several oligonucleotide(s). Such a mechanism is an exo-endonucleolytic one, provided that one uses the

definition of an exonuclease previously given by Paoletti *et al.* (1971). "An enzyme which catalyzes a stepwise attack beginning either at the termini of a DNA molecule or at single-strand interruptions within the molecule, sequentially liberating mono- or oligonucleotides."

The association of an exonucleolytic mechanism of action with an endonucleolytic has already been demonstrated for the *Micrococcal* nuclease (Williams *et al.*, 1961) and for *E. coli* endonuclease I (Paoletti *et al.*, 1971). It must be noted that the latter enzyme yields products whose average size is similar to that of the products of the *Helix* enzyme.

One can argue that no oligonucleotide whose average length ranges around 6.4 is detected during the initial stage of attack of DNA by *Helix* DNase. However, it can be shown that the amount of such oligonucleotides, which could have been eventually liberated by the exo-endonucleolytic mechanism, is below the threshold of their detection in the sedimentation experiments which lies around 2% of the total material.

During the time elapsed in our longest experiments (20 min) (Figure 4C) each DNA molecule has received on the average around 100 endonucleolytic breaks as established from the fluorometric data. If each endonucleolytic break were accompanied by the removal of a short piece of oligonucleotides whose average length is 8, $100 \times 8 = 800$ nucleotides would have been removed during this period. This corresponds to only 800/90,000, *i.e.*, less than 1% of total DNA which is a nondetectable amount of radioactivity. Experiments devised for directly establishing the exo-endonucleolytic mode of action of *Helix* endonuclease are in progress.

From the pioneering effort of Laskowsky (1961, 1967) to define rules for the classification of nucleases, the only criteria which remains unambiguous is the cleavage of the internucleotide bond on the 3'-phosphate *vs.* the 5'-phosphate side. *Helix* enzyme is a 3'-phosphomonoester former, a property which seems so far to be a characteristic of acid DNase: spleen, thymus ones (Bernardi, 1968), *Bothrops atrox* venom (Georgatsos and Laskowski, 1962). To our knowledge, the only exception to this rule could be an endonuclease extracted from *Octopus vulgaris*, active at pH 6.0, which yields 3'-phosphate ends (Georgatsos and Antonoglou, 1966). A nuclease from *Micrococcus pyogenes* yields also 3'-phosphate oligonucleotides, in spite of being active at alkaline pH. But this enzyme is nonspecific for the sugar (Cunningham *et al.*, 1956).

The rate of attack on closed DNA circles is not affected by twisting. Therefore, the affinity of the enzyme for the circles is not modified by the existence of an excluded volume. Moreover, the modification of the secondary structure of the supercoils (Dean and Lebowitz, 1971) is not recognized by *Helix* endonuclease. An identical independence of enzyme action toward the supercoiling has also been found for pancreatic DNase and beef spleen acid DNase (our unpublished results). These observations should be brought together with those of Mitsui *et al.* (1970), obtained on left-hand polynucleotides, showing that twelve different enzymes of nucleic acid metabolism, among which are pancreatic DNase, exonuclease I and II of *E. coli*, micrococcal nuclease, spleen phosphodiesterase, and *M. luteus* exonuclease, do not recognize left-handed polynucleotides from the natural right-handed ones.

These data indicate that most of known nucleases recognize only an extreme change of the secondary structure (native *vs.* denatured). They contrast with an observation reported by Hayashi and Hayashi (1971). These authors compared the allomorphic forms of the replicative form (RF DNA) of ϕ X-

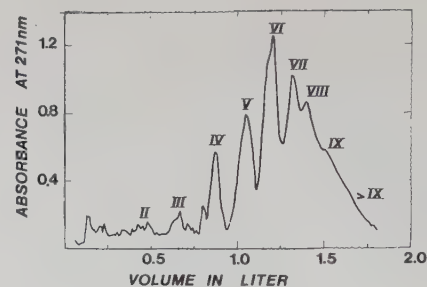


FIGURE 6: DEAE-cellulose chromatogram in 7 M urea of the exhaustive digest of calf thymus DNA by *Helix* DNase. A DEAE-cellulose column (2.5 $\times$ 27 cm) is loaded with 475 OD₂₇₁ units. A linear gradient is set between 0 and 0.3 M NaCl in 0.1 M sodium acetate (pH 4.7) at 25°. Roman numerals give the lengths of oligonucleotides determined as described in Methods. Digestion conditions are given in Table II. Recovery of absorbing material is 99% of the absorbance of the input.

174, for their template activity, *i.e.*, the rate of *in vitro* synthesis by *E. coli* DNA-dependent RNA polymerase. Twisted RFI DNA which does not have a break on either strand, has a rate of transcription which is about three times higher than that of untwisted RFII DNA, derived from RFI by introducing a single-strand break on either strand by pancreatic DNase.

Acknowledgments

The authors wish to thank Dr. G. Bernardi for critical interest, Dr. F. Laval for help during the course of this work, Dr. J. B. Le Pecq and Dr. A. Jacquemin-Sablon for stimulating discussions, and Dr. C. Delaporte and Miss E. Franque for their excellent technical assistance at different stages of the work.

References

- Bernardi, G. (1968), *Advan. Enzymol.* 31, 1.
- Bernardi, G., and Grifffé, M. (1964), *Biochemistry* 3, 1419.
- Britten, R. J., and Kohne, D. E. (1968), *Science* 161, 529.
- Carrara, M., and Bernardi, G. (1968), *Biochemistry* 7, 1121.
- Cunningham, L., Catlin, B. W., Privat de Garilhe, M. (1956), *J. Amer. Chem. Soc.* 78, 4642.
- Dean, W. W., and Lebowitz, J. (1971), *Nature (London), New Biol.* 231, 5.
- Freifelder, D., and Trumbo, B. (1969), *Biopolymers* 7, 681.
- Georgatsos, J. G., and Antonoglou, O. (1966), *J. Biol. Chem.* 241, 2151.
- Georgatsos, J. G., and Laskowski, M., Sr. (1962), *Biochemistry* 1, 288.
- Hayashi, Y., and Hayashi, M. (1971), *Biochemistry* 10, 4212.
- Jollès, P. (1964), *Angew. Chem. Int. Ed. Engl.* 3, 28.
- Kunitz, M. (1949), *J. Gen. Physiol.* 33, 349.
- Laskowski, M., Sr. (1961), *Enzymes* 5, 123.
- Laskowski, M., Sr. (1967), *Advan. Enzymol.* 29, 165.
- Laval, F., Malaise, E., and Laval, J. (1970), *Exp. Cell. Res.* 63, 69.
- Laval, J., and Paoletti, C. (1972), *Biochemistry* 11, 3596.
- Lehman, I. R., Roussos, G. G., and Pratt, E. A. (1962), *J. Biol. Chem.* 237, 819.
- Martin, J. B., and Doty, D. M. (1949), *Anal. Chem.* 21, 965.
- Mitsui, Y., Langridge, R., Shortle, B. E., Cantor, C. R., Grants, R. C., Kodama, M., and Wells, R. D. (1970), *Nature (London)* 228, 1166.

- Paoletti, J., and Le Pecq, J. B. (1971), *Eur. Biophys. Congr. Proc., Protein, Nucleotides, I*, 391.
- Paoletti, C., Le Pecq, J. B., and Lehman, I. R. (1971), *J. Mol. Biol.* 55, 75.
- Philipson, L., Albertson, P. A., and Frick, G. (1960), *Virology* 11, 553.
- Revet, B. M. J., Schmir, M., and Vinograd, J. (1971), *Nature (London), New Biol.* 229, 10.
- Richardson, C. C. (1965), *Proc. Nat. Acad. Sci. U. S.* 54, 158.
- Richardson, C. C. (1966), *J. Mol. Biol.* 15, 49.
- Richardson, C. C., Inman, R. B., and Kornberg, A. (1964), *J. Mol. Biol.* 9, 46.
- Schumaker, V. N., Richards, E. G., and Schachman, H. K. (1956), *J. Amer. Chem. Soc.* 78, 4230.
- Signer, R., and Schwander, H. (1949), *Helv. Chim. Acta* 32, 853.
- Studier, F. W. (1965), *J. Mol. Biol.* 11, 373.
- Thomas, C. A., Jr. (1956), *J. Amer. Chem. Soc.* 78, 1861.
- Tomlinson, R. V., and Tener, G. M. (1963), *Biochemistry* 2, 697.
- Williams, E. J., Sung, S. C., and Laskowski, M. (1961), *J. Biol. Chem.* 236, 1130.

